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Inhibition of exopolysaccharide biopolymer and pyocyanin virulence factors produced by *Pseudomonas aeruginosa* 1604 by salicylic compounds

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RESEARCH ARTICLE

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Abstract

Pseudomonas aeruginosa is able to form exopolysaccharide biopolymer in the lungs of cystic fibrosis patients, increasing viscoelasticity of sputum. In our studies, the viscosity of the biopolymer produced by P. aeruginosa 1604 was investigated in the presence of aspirin and sodium salicylate. The applied salicylic compounds in 0.3 % concentration decreased significantly the viscosity of the biopolymer during a five days period. The biopolymer of P. aeruginosa can harbour other virulence factors, such as pyocyanin. The applied salicylic compounds also decreased the pyocyanin production; however, according to our results P. aeruginosa 1604 was able to produce pyocyanin after 72 h in the presence of 0.1-0.2 % salicylic compounds. Consistent low pyocyanin production was caused only in the presence of 0.3 % or higher concentration of these compounds.

Consequently, salycilic compounds decreased both the viscoelasticity of the biopolymer and the concentration of pyocyanin produced by P. aeruginosa 1604.

Keywords

Pseudomonas aeruginosa · exopolysaccharide biopolymer · pyocyanin · aspirin · sodium salicylate

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1 Introduction

P. aeruginosa is a wide spread opportunistic pathogen bacterium, which presences in the lung and sputum of patient suffering from cystic fibrosis. Cystic fibrosis is a clinical syndrome characterized by chronic sinopulmonary infection, gastrointestinal, nutritional, and other abnormalities. Chronic *P. aeruginosa* infection causes epithelial surface damage and airway plugging, which results decreased pulmonary function [1]. Moreover, *P. aeruginosa* plays role in superinfection of serious burn wounds and ulcer [2,3]. *P. aeruginosa* can form biofilms on biotic surfaces, such as in the lungs of cystic fibrosis patients triggering infections that are extremely difficult to eradicate [4].

P. aeruginosa growing in biofilms are less susceptible to bacteriophage, to amoebae, and to the chemically diverse biocides or antibiotics used to combat biofouling in industrial processes or in human therapy.

P. aeruginosa strains have different genotypes [5], as well as phenotypes. The mucoid bacterial cells, which attached in biofilm, can withstand host immune responses compare to the non-attached individual planktonic cells [6]. Firstly, patients are colonized by non-mucouid *P. aeruginosa*, however, as the disease progresses mucoid strains occur. In bacterial biofilm, there are bacterial cells enclosed in a polymeric matrix produced by the bacteria [7]. The mucoid exopolysaccharide material of *P. aeruginosa* strains is mainly alginate, which is a heteropolymer of mannuronic and guluronic acid derivatives. This exopolysaccharide protects the bacterial cells due hindering antibiotics to reach cell surface and thus contributing to the antibiotic resistance of mucoid strains [8]. Furthermore, alginate produced by mucoid cells decreases the uptake and early antibacterial effect of aminoglycosides [9]. Alginate increases the bacterial colonization within the respiratory tract by increasing their adherence to the respiratory epithelia [10]. Alginate also increases viscoelasticity of properties of sputum secreted by cystic fibrosis (CF) patient [11].

The increase stiffness of the sputum hinders the proper clearance of fluid from the airway; therefore therapeutic methods are often directed to decreasing the viscoelastic parameters of

this fluid. CF sputa are typically highly viscous and elastic and are removed with difficulty by the ciliary and cough mechanism. Therefore, decreasing stiffness of CF sputum is a major goal for treatment of this disease, and rheological characterization of these materials can facilitate to modify viscoelasticity in the diseased state [12].

Furthermore *P. aeruginosa* caused serious problems include its ability to develop resistance to antibiotics, release a large scale of extracellular virulence factors, like elastase, alkaline protease, hemolysin, rhamnolipids and pyocyanin [13]. Pyocyanin alters Ca^{2+} homeostasis in human airway epithelial cells, including inhibition of the response to Ca^{2+} agonists, by this pyocyanin could interfere with critical host defense mechanisms contributing the symptoms in *Pseudomonas* associated lung disease [14]. Salicylic acid and its derivatives are nonsteroidal anti-inflammatory drugs that inhibit growth and biofilm formation of *P. aeruginosa* and *S. epidermidis* and also downregulate some of the virulence factors in *P. aeruginosa*. They have inhibitory effects both as a sole agent and in combination with antibiotics. It has been reported that salicylic acid in vitro reduced the attachment of these bacteria to human corneal epithelial cells. Furthermore, salicylic acid has been shown to alleviate the virulence of *P. aeruginosa* on *Arabidopsis thaliana* and *Caenorhabditis elegans* [15].

Salicylic acid is known as a phenolic metabolite produced by plants. In plants salicylic acid plays an important role in the induction of plant defence responses against pathogen attack. Salycilic acid can reduce attachment and biofilm formation of *P. aeruginosa* on the roots of the *Arabidopsis*, however above a given concentration did not inhibit its growth. Furthermore, salicylic acid completely inhibited the aerobic biofilms, however it did not decrease the formation of anaerobic biofilm [16].

The affect of salicylic acid and its derivates on pyocyanin production of *P. aeruginosa* was generally monitored at low concentrations (0.1-1 mM) or in a specified concentration, but only for a short period (24 hours) [15,16]. Furthermore the formed biofilm was characterized based on its vitality and thickness [16,17,18]. However, these methods could not provide information about the long-term affect and the necessary inhibitory concentration of salicylic compounds and about the structure of exopolysaccharide biofilm. Therefore, the aim of present study is to investigate the effect of two salicylic compounds, aspirin and sodium salicylate, on rheological properties of exopolysaccharide biopolymer and pyocyanin production of the non-clinical origin *Pseudomonas aeruginosa* 1604 in a long-term (120 hours) period.

2 Materials and Methods

2.1 Bacteria strain and culture conditions

P. aeruginosa 1604 strain isolated from reservoir water of a Hungarian oil well product was cultured in Bouillon medium (10 g peptone, 3 g Beef extract, 4 g $Na_2HPO_4 \cdot 2H_2O$, 3 g NaCl, pH adjusted to 7.2) for 24 hours at 37 °C in a shaker at 180 rpm.

2.2 Effect of salicylic acid/aspirin on biopolymer formation

The biopolymer was produced in modified Bouillon medium (10 g peptone, 3 g Beef extract, 2 g KH_2PO_4, 3 g NaCl, 8 g NH_4NO_3, 10 g sucrose, 1 g glucose, 5 % glycerol-rich product (30 % saponified palmitic acid and stearic acid, 62 % glycerol, 1 % methanol, 7 % metallic salts)) at 37 °C, which was inoculated with *P. aeruginosa* 1604 grown in Bouillon medium for 24 hour. According to our previous experiences in this medium the biopolymer production of *P. aeruginosa* 1604 is significantly enhanced compared to the original Bouillon medium. The final structure of biopolymer was formed after 5 days.

The biopolymer production was also investigated in the presence or absence of salicylic acid or aspirin. The concentrations of these compounds were in the range of 0.1 to 0.4 %.

2.3 Biopolymer characterization

The characterization of biofilm was studied by rheological method. The rheology of biopolymer was measured by using a Brookfield DV-II+ Viscometer. The biopolymer samples were measured after 5 days at 37 °C. The shear rate (s^{-1}) vs. shear stress (Nm^{-2}) curve was used in this study to characterize the flow ability of biopolymer. Since the characteristic of bacterial biopolymer is viscoelastic and the applied viscometer is appropriate for viscous substances, data was calculated as the ratio of shear stress (Nm^{-2}) to shear rate (s^{-1}), which means the apparent viscosity [19]. Due to the inhomogeneous structure of the biopolymers (viscous-elastic gel or liquid depending on the concentration) the calculation of apparent viscosity was necessary.

2.4 Quantification of produced biopolymer

All of the biopolymer samples (25 ml) were precipitated with 1 % $CaCl_2$ solution. During the process the systems were shacked for 4 hours, after that they were left stand overnight. The samples were filtrated and dried on 50 °C for one day and dry weight was determined. A blank measurement was also generated, that contained all the compounds of Bouillon-B medium and *P. aeruginosa* 1604 cells at $1.8 \cdot 10^7$ $CFUml^{-1}$ concentration (in this sample biopolymer synthesis did not take place). The blank sample was also treated with 1 % $CaCl_2$ solution in order to precipitate those macromolecules that able to interact with Ca^{2+} (besides biopolymer). The average of measured dry weight (g) values was referred to 100 ml biopolymer solution and data were added in w/v %.

2.5 Inhibition of pyocyanin production

The pyocyanin was produced in modified KingA medium (10 g whey powder, 1.4 g magnesium-chloride, 10 g potassium-phosphate, 10 g sodium-citrate) at 37 °C, which was inoculated with *P. aeruginosa* 1604 grown in Bouillon medium for 24 hour. The amount of bacterial biomass was monitored by

Hach-Lange DR 5000 photometer at 600 nm. The pyocyanin production was investigated in the presence of salicylic acid or aspirin as well. The concentrations of these compounds were in the range of 0.1 to 0.4 %. The pyocyanin was produced in modified KingA medium also in these cases.

2.6 Quantification of pyocyanin

The pyocyanin production was investigated by a photometric method after extraction according to Essar et al. [20]. A 5 ml sample of culture to maximize pyocyanin production was extracted with 3 ml of chloroform and re-extracted into 1 ml of 0.2 N HCl to give a pink to deep red solution. Concentrations of pyocyanin in the culture supernatant were determined based on the absorbance at 520 nm (Hach-Lange DR 5000 photometer) and expressed in µg ml^{-1} calculating with its extinction coefficient ($2.46 \cdot 10^3$ M^{-1} cm^{-1}, in 0.2 N HCl at 520 nm) [21].

3 Results

3.1 Rheological measurement

During the investigation of rheology properties of biopolymer produced by *P. aeruginosa* 1604, it was found that the shear stress of the produced biopolymer was almost 45 Nm^{-2} and the apparent viscosity was 1.97 Nsm^{-2} at 22 sec^{-1} shear rate. Based on the results, the investigated bacterium strain could produce high viscosity biopolymer during a five days period under appropriate conditions.

Figure 1 shows the changes of rheological features of the medium in the presence of aspirin (A) or sodium salycilate (B) during the biopolymer production compared to a control system.

Based on the flow curves, the viscosity of the biopolymer decreased in case of growing concentration of salicylate compounds. In the presence of 0.1 and 0.2 % aspirin the biopolymer had viscous property (0.64 and 0.69 Nsm^{-2} at 22 sec^{-1} shear rate), while at 0.3 and 0.4 % concentration the apparent viscosity was 0.25 and 0.16 Nsm^{-2} at 22 sec^{-1} shear rate and the maximum shear stress decreased tenfold compared to the control. It is interesting, that 0.2 % aspirin reduced less the viscosity compared to 0.1 %; however, the differences in the effect between these concentrations is not significant.

Sodium salicylate caused similar changes in the viscous properties of the biopolymer. In this case, significant decreasing could be observed in the presence of 0.2 % sodium salicylate (0.22 Nsm^{-2} at 22 sec^{-1}). Furthermore, the growing concentration resulted in lower viscosity as it shown on the flow curves, apparent viscosity was 0.10 Nsm^{-2} at maximum shear rate and the maximum shear stress decreased tenfold at 0.4 % sodium salicylate

Consequently, the investigated salicylic compounds could inhibit effectively the exopolysaccharide production of *P. aeruginosa* 1604; thereby these can be applicable for therapeutic targets, for example treatment of cystic fibrosis.

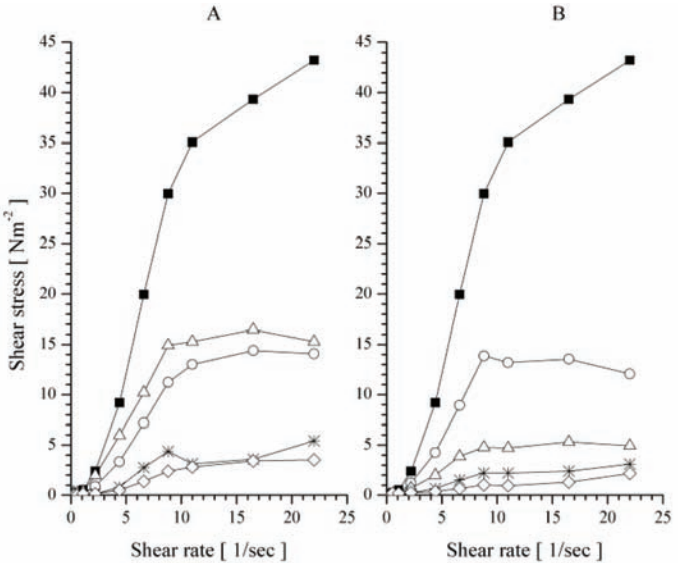

Fig. 1. Changes in the rheological features of biopolymer produced by *P. aeruginosa* 1604 in the presence of aspirin **(A)** and sodium salicylate **(B)** in different concentrations; ■ = control (0 %), ○ = 0.1 %, Δ = 0.2 %, * = 0.3 %, ◊ = 0.4 %.

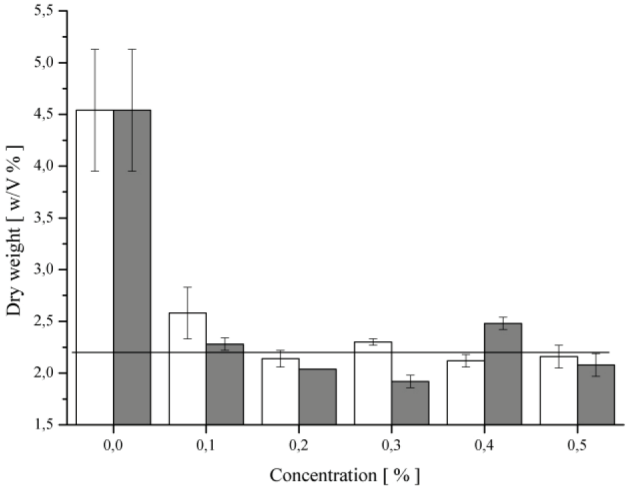

Fig. 2. Changes in the amount of biopolymer produced by *P. aeruginosa* 1604 in the presence of salicylic compounds; Line = blank, white column = dry weight of aspirin, grey column = dry weight of sodium salicylate.

3.2 Quantification of produced biopolymer

Besides rheological properties, amount of the formed biofilm was measured as well. Therefore biopolymer samples produced during five days under appropriate conditions were coagulated by 1 % $CaCl_2$ solution. The pellet of the precipitation was dried to constant weight.

As *P. aeruginosa* 1604 proved to be a mucoid strain, its biopolymer production was detected in modified Bouillon medium in the presence and absence of salicylic compounds after 5 days (Figure 2). Significant dry weight values (4.54 ± 0.59 %) were found in the absence of sodium salicylate and aspirin compared to the blank. The blank (line) shows the

amount of macromolecules, which could be precipitated by $CaCl_2$, however, they could not form viscoelastic biopolymer structure.

The results also revealed that the applied aspirin or sodium salicylate at 0.1 % concentration were almost totally able to inhibit the synthesis of macromolecules responsible for alginate biopolymer formation. The amounts of the precipitated pellet were almost the same as the blank line 2.2 ± 0.12 %. Significant differences in the effect of aspirin and sodium salicylate were not detected according to the data.

The results referred to the efficiency of the applied salicylic compounds in the inhibition of exopolysaccharide-type biopolymer of *Pseudomonas aeruginosa*.

3.3 Inhibition of pyocyanin production

The other most important virulence factor of *P. aeruginosa* is pyocyanin beside exopolysaccharide production. Pyocyanin is an amphoter molecule, which is the basis of its detection. The pyocyanin production was investigated by a photometric method after extraction.

Before investigation of the inhibition, the production rate of pyocyanin and growth of biomass was monitored depending on time.

According to our results the growth of biomass reached the maximum (OD_{600} is 1.47) after 72 hours, while pyocyanin production was in the exponential phase in this time. Therefore investigation of pyocyanin production in longer (120 hours) period is proved to be important, instead of the generally monitored 24-48 hours [16].

It was found that pyocyanin, produced by *P. aeruginosa* 1604 was significantly lower in the presence of aspirin or sodium salicylate. Aspirin and salicylate decreased pyocyanin production by 58 % and 65 % after 120 hours as shown in the Figure 4. However, in the presence of 0.1 % aspirin or sodium salicylate, *P. aeruginosa* 1604 was still able to produce considerable amount (0.44 μgml^{-1}) of pyocyanin after a 72 hours lag period. A constant suppression of pyocyanin production can be achieved using 0.3 % or higher concentration of these compounds.

According to our results, the applied salicylic compounds could inhibit not only biofilm forming of *P. aeruginosa*, but also they could decrease effectively the production of pyocyanin, which is one of the most important virulence factors.

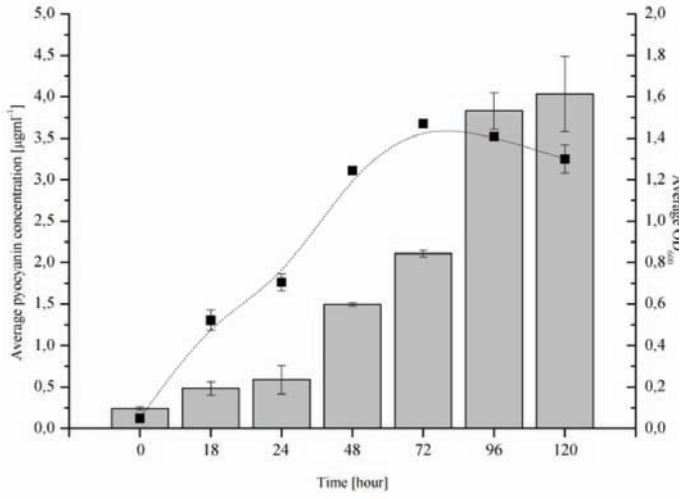

Fig. 3. Growth and pyocyanin production of *P. aeruginosa* 1604; Light grey columns = average pyocyanin concentration, black squares = average OD_{600} value.

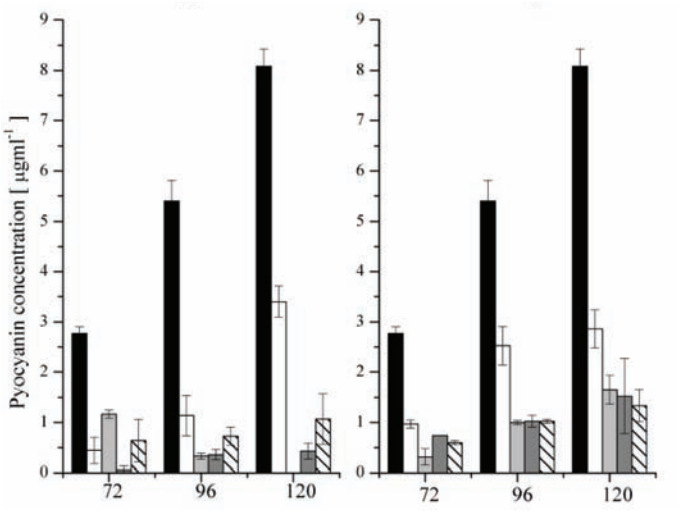

Fig. 4. Changes in the amount of pyocyanin in the presence of aspirin **(A)** and sodium salicylate **(B)** in different concentrations, black columns = control (0 %), white columns = 0.1 %, light grey columns = 0.2 %, grey columns =0.3 %, striped columns = 0.4 %.

4 Discussion

According to Prithiviraj et al. [16] some virulence factors of *P. aeruginosa* were inhibited by salicylic compounds [16], as well as *P. aeruginosa* 1604 originated from non-clinical condition. Altough the inhibition effect on biopolymer and pyocyanin synthesis was detectable in the case of both two salicylic compounds, aspirin and sodium salicylate had got different efficiency in these processes. In the case of inhibition of biopolymer synthesis, sodium salicylate was more effective than aspirin based on apparent viscosity, since at 0.2 % concentration and 22 sec^{-1} shear rate the apparent viscosity were 0.22 and 0.69 Nsm^{-2} respectively the two compounds. However aspirin could inhibit more effectively the pyocyanin synthesis in 0.3 and 0.4 % concentrations.

The physical properties of exopolysaccharide biopolymer produced by *P. aeruginosa* had essential importance in infection processes. Investigation of the thickness of created biofilm [18] can not provide information about its structure and mechanical resistance. The rheological measurements clearly demonstrated that the investigated salicylic compounds in appropriate concentrations could negatively influence the structure of synthesized biopolymer.

In previous studies the effect of salicylic compounds for *P. aeruginosa* was investigated at low concentrations for short

period [15,16] However our results demonstrated, that under appropriate grow conditions, the *P. aeruginosa* is able to survive in the presence of salicylic compounds for long-term. Altough these salicylic compounds caused metabolic disorder in short-term, however *P. aeruginosa* 1604 could produce pyocyanin or biopolymer in a longer period. Furthermore for maintainence of the inhibition for longer period, significantly higher concentrations were necessary.

All these results referred to the possibility of clinical applications of aspirin and sodium salicylate in case of *P. aeruginosa* infections for example in CF, burn wounds and ulcers. Altough for the long-term inhibitions higher amount of applied salicylic compounds (0.3 and 0.4 %) are necessary, these concentrations generally applied in therapeutic practise in case of adults.

5. Conclusions

We successfully used sodium salicylate and aspirin in different concentrations to inhibit pyocyanin and biopolymer production of *Pseudomonas aeruginosa* 1604. The amount of biopolymer has decreased significantly after five days. Inhibition of pyocyanin production was investigated only in a 24h period previously [16]; in our study we have found that the long term inhibition can be achieved, but requires higher concentration of salicylic compounds: at 0.3 % concentration the inhibitory effect was evident for 120 hours.

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